Interdependence of Coenzyme-Induced Conformational Work and Binding Potential in Yeast Alcohol and Porcine Heart Lactate Dehydrogenases: A Hydrogen–Deuterium Exchange Study†

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ABSTRACT: Binding of NAD coenzymes to yeast alcohol dehydrogenase (YADH) and porcine heart lactate dehydrogenase (PHLDH) was studied by hydrogen–deuterium exchange with the infrared technique. Conformational changes in the enzymes specific to the coenzymes and their fragments were observed, and the pH dependence of the exchange reaction shows that it conforms to the EX-2 scheme. In both YADH and PHLDH the magnitude of the conformational change as measured by exchange retardation is considerably larger for NAD^+ than for NADH. Studies with coenzyme fragments like ADP-ribose, ADP, and AMP also highlight the lack of rigorous correlation between structural features such as charge and size and their influence on exchange behavior. Ternary complexes such as YADH–NAD^+–pyrazole, PHLDH–NAD^+–oxalate, and PHLDH–NADH–oxamate, which mimic the transition state, have a significantly more pronounced effect on exchange rates than the corresponding binary complexes. The outstanding feature of this study is the demonstration that in the binary enzyme–coenzyme complexes the more loosely bound NAD^+ is more effective in retarding exchange than the more firmly bound NADH. These differences are attributed to the unequal structural constraints exerted by the two coenzymes upon the enzymes, which translate to unequal expenditure of transconformational work in the formation of the two complexes. The opposing variation in the free energy of binding and the transconformational work expended can be viewed as an unequal partitioning of the net free energy gain resulting from the protein–ligand interaction into a binding term and that required for conformational change.

Ligand-induced conformational changes have been demonstrated in several of the pyridine nucleotide dependent dehydrogenases. X-ray crystallographic studies on DFLDH[1] and LADH[1] have shown that coenzymes and coenzyme fragments do not necessarily bind in the same manner to the active site and that, depending on the ligand, the protein can assume quite specific structures in the complex [Brändén and Eklund (1978) and references cited therein; Adams et al., 1973; Chandrasekhar et al., 1973]. One of the most sensitive methods available for probing the structural dynamics of proteins and the effects of ligand binding on these processes is the measurement of the exchange of interior peptide hydrogens with the solvent (Englander & Kallenbach, 1984). Numerous studies have shown that the accessibility of peptide hydrogens is influenced both by the inherent stability of the folded polypeptide structure and also by the constraints imposed by complex formation with ligands (Woodward & Hilton, 1979). Measurements of the time course of exchange have thus far been employed to study the interaction of enzymes with coenzymes, prosthetic groups, metal ions, substrates, and inhibitors, and more recently the method has been shown to be suitable for detecting conformational changes in an enzyme that accompany the catalytic process (Ottesen, 1971; Anzai et al., 1981; Zavodszky et al., 1966; Stryker & Parker, 1970; Weisshaar & Palm, 1972; Nabedryk-Viala et al., 1977; Ohta et al., 1978; Printz & Gounaris, 1972; Wickett et al., 1972; Woodward, 1977; Browne & Waley, 1974; Deluca & Marsh, 1967; Takahashi & Westhead, 1971; Mizuta et al., 1980; Pfister et al., 1978). Such specific differences have in the past also been observed in the H–D exchange[1] kinetics of complexes of various dehydrogenases with NAD and NADH (Hvidt & Kägi, 1963; DiSabato & Ottesen, 1966; Zavodszky et al., 1966; Stryker & Parker, 1970).

In this study we have elaborated upon the exchange behavior of the coenzyme and coenzyme-fragment complexes of YADH[1] and PHLDH.[1] The data corroborate that NAD^+ and NADH differ very substantially in their effectiveness to restrict the H–D exchange of the proteins. In fact, they reveal an inverse relationship between the stability of the enzyme–coenzyme complex and the effectiveness of the coenzyme to tighten the protein structure as gauged by H–D exchange measurements. The results are taken to suggest that the isotopic exchange method can serve as a tool to assess semiquantitatively the energetics of ligand-induced changes in conformation. They also imply the participation of intramolecular transconformational processes in the fine tuning of ligand-binding equilibria associated with enzyme catalysis and metabolic requirements.

Materials and Methods

YADH was purchased as a lyophilized powder from Boehringer. PHLDH was obtained from Sigma as a crystalline suspension in 1.9 M ammonium sulfate, pH 6. NAD^+ and NADH were obtained from Boehringer. ADP-ribose, ADP, and AMP were from Sigma, and pyrazole was from Aldrich. Acetone and 2-propanol for UV spectroscopy were purchased from Fluka. Oxalate was purchased as potassium

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[1] Abbreviations: YADH, yeast alcohol dehydrogenase; LDH, lactate dehydrogenase of unspecified origin; DFLDH, dogfish lactate dehydrogenase; PHLDH, porcine heart lactate dehydrogenase; CHLDH, chicken heart lactate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LADH, liver alcohol dehydrogenase; GDH, glutamate dehydrogenase, MDH, malate dehydrogenase, H–D exchange, hydrogen–deuterium exchange.

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salt from Merck and oxamate as sodium salt from Aldrich. ADP was obtained as barium salt and was converted to the sodium form as follows: 300 mg of barium salt was mixed with 2 mL of AG 50 W-X2 cation-exchange resin (Bio-Rad; 200–400 mesh, hydrogen form). ADP, hydrogen form, was recovered by washing the suspension with 2 mL of H_2O and was neutralized with NaOH to pH 7.5. D_2O was purchased from Medipro, Teufen, Switzerland. NaOD (40% in D_2O) and DCl (38% in D_2O) were products of Fluka.

Preparation of Samples. A 200–400-mg quantity of enzyme was dissolved in an appropriate volume of a buffer solution containing 0.75 mM NaH_2PO_4 and 4.25 mM Na_2HPO_4, pH 7.5, to give a 10% solution which was dialyzed for 24 h against three changes of a 1000-fold volume of the same buffer. Insoluble material was removed by centrifugation at 25000*g* for 30 min at 4 °C and the concentration adjusted to 30 mg of protein/mL. Enzyme concentration was measured spectrophotometrically at 280 nm with $E^{1\%}_{1cm}$ = 12.6 for YADH (Hayes & Velick, 1954) and $E^{1\%}_{1cm}$ = 14.0 for PHLDH (Holbrook et al., 1975). Activities were measured by the methods of Hoch and Vallee (1958) and Hohorst (1970). The specific activities were usually 280 units mg^{-1} for YADH and 260 units mg^{-1} for PHLDH.

For the exchange studies 200-μL volumes of enzyme (6 mg) were pipetted into individual microvessels (Buch and Holm, Copenhagen), frozen in liquid nitrogen, and placed over P_2O_5 in a vacuum desiccator that was evacuated to 10^{-2}–10^{-1} Torr (Hvidt et al., 1960). After lyophilization for 24 h at room temperature, the vacuum was released and the microvessels were sealed with greased caps to prevent uptake of water vapor during storage at −20 °C up to 21 days prior to the exchange experiments. The specific activity of the enzymes remained constant over this period. To minimize the variations introduced by the preparation of the protein, all comparative experiments were carried out on samples prepared from the same batch of enzyme and lyophilized at the same time from the same solution. Buffered solutions of D_2O were prepared by dissolving 0.15 mmol of $NaH_2PO_4 \cdot H_2O$ and 0.85 mmol of $Na_2HPO_4 \cdot 2H_2O$ in 10 mL of D_2O (pD 8.0), by dissolving 0.67 mmol of $NaH_2PO_4 \cdot H_2O$ and 0.33 mmol of $Na_2HPO_4 \cdot 2H_2O$ in 10 mL of D_2O (pD 7.0), and by dissolving 1 mmol of $Na_4P_2O_7$ in a final volume of 10 mL of D_2O after neutralization of the solution with NaOD in D_2O to a pD of 9.0. pD was obtained from the relationship pD = pH (meter reading) + 0.4 (Glashoe & Long, 1960). Solutions of 2-propanol (39 mM), acetone (38 mM), and pyrazole (5 mM) in buffered D_2O were prepared by direct addition.

Solutions of NAD^+ (50 mM), coenzyme fragments (50 mM), potassium oxalate (1 mM), and sodium oxalate (1 mM) in D_2O were prepared by lyophilizing 1–2 mL of aqueous solutions that had been adjusted to pH 7.5. Solutions of NADH (50 mM) did not require this adjustment. To remove all exchangeable hydrogens, the lyophilized samples were redissolved in a minimum volume of D_2O, lyophilized again for another 24 h, and stored protected from water vapor as described above. To avoid the formation of inhibitors from NADH (Kaplan et al., 1961), solutions of this coenzyme (50 mM) in D_2O were prepared before each exchange experiment by twice lyophilizing a freshly made aqueous solution.

Exchange Experiments. H–D exchange was measured by infrared spectroscopy (Ottesen, 1971) with a Beckman IR 20A double-beam grating infrared spectrometer. Calcium fluoride cells of 0.10-mm path length were employed. Exchange experiments were started by pipetting 200 μL of D_2O containing 0.1 M sodium phosphate buffer with or without other constituents into microvessels in which 6 mg of YADH had been previously lyophilized. The starting time of the exchange experiment was taken as 30–60 s after the addition of D_2O solution, the time required for solubilization being somewhat variable. Upon dissolution the sample was transferred into the calcium fluoride IR cell. The second cell was filled with the reference solution, which except for the enzyme had the same composition as the first cell. The first measurement was made 3–4 min after the beginning of the exchange experiment. Data were taken on the Beckman IR spectrophotometer in the double-beam mode, with full-scale deflection adjusted to 0.95 of the chart width. The base line was recorded from 1700 to 1400 cm^{-1} by filling both cells, reference and sample, wih the reference solution.

The exchange reaction was followed by repetitive measurements of the transmittance at the amide II and amide I bands of the protein at 1545 and 1645 cm^{-1} for YADH and 1552 and 1658 cm^{-1} for PHLDH, respectively. Each set of measurements requiring 30–60 s was repeated every 15 min during the first hour of the exchange reaction and every 30 min for the next 4 h. To minimize changes in temperature, the cells were removed from the beam between measurements and placed on a metal plate thermostated at 20 ± 0.5 °C. The spectra were recorded on a linear transmittance scale. The absorbance of the sample was corrected for the absorbance of the corresponding reference solution at each frequency.

Calculation of Number of Unexchanged Hydrogens. The change in concentration of the peptide hydrogens corresponding to a given change in intensity of the NH absorption band was calculated from the IR spectrum of solid lyophilized YADH recorded in a potassium bromide pellet and of a sample of YADH in D_2O in which, by treatment with 1% sodium dodecyl sulfate at 70 °C for 7 h, all peptide hydrogens were presumed to be exchanged. The ratio of the absorbance of the amide II band (A_{II}) to the amide I band (A_I) was 0.11 for the deuteriated protein and 0.65 for the solid nondeuteriated sample. By relating the difference between the measured ratio (A_{II}/A_I) and the residual absorbance ratio of the fully exchanged protein to the maximum difference of the ratios of 0.54, one obtains for the percentage of unexchanged peptide hydrogens

$$\% \text{ NH unexchanged} = [(A_{II}/A_I) - 0.11] \times 100/0.54$$

The number of unexchanged peptide hydrogens equals fraction unexchanged times 1388, where 1388 corresponds to the number of amino acid residues per mole of YADH (Sund & Theorell, 1963). Since the A_{II}/A_I ratio of unexchanged and of fully exchanged PHLDH was not determined, the results obtained with this enzyme are given simply as the measured A_{II}/A_I values.

Exchange Studies in the Presence of Coenzymes, Coenzyme Fragments, and Inhibitors. The degree to which coenzymes and coenzyme analogues were bound is evaluated as a function of total concentration of enzyme and coenzyme according to the relationship (Hvidt & Kägi, 1963)

$$[C_b] = \{K' + 4[P] + [C_t] - (K' + 4[P] + [C_t]^2 - 16[P] \times [C_t])^{1/2}\}/2$$

where K' is the apparent dissociation constant, $[C_t]$ and $[C_b]$ are total and bound concentrations of the coenzymes, and [P] is the total concentration of the enzyme. K' was taken as 6.7×10^{-4} M for the YADH–NAD^+ complex, as 2.5×10^{-5} M for the YADH–NADH complex, and as 1.3×10^{-3} M and 2×10^{-3} M for the complexes of YADH with ADP-ribose and ADP, respectively, as measured by spectropolarimetric titration in 0.1 M sodium phosphate buffer, pH 7.5 (Kägi, unpublished

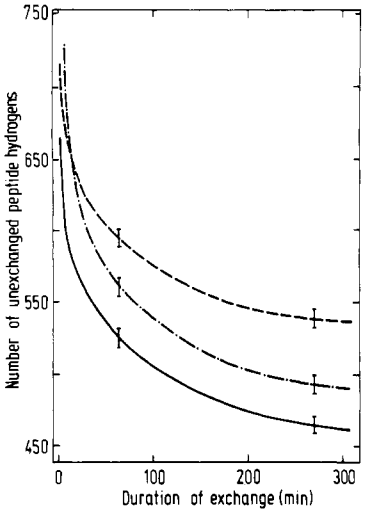

FIGURE 1: H–D exchange of YADH and YADH–coenzyme complexes at pD 8.0. Sample compositions: YADH (—); YADH + NADH (5×10^{-2} M) (–·–); YADH + NAD^+ (5×10^{-2} M) (- - -). [Enzyme], 2×10^{-4} M. The error bars indicate the standard deviation determined from the variation of three consecutive exchange experiments carried out on samples of the same batch of enzyme and lyophilized simultaneously from the same solution.

observations). For the YADH–AMP complex the value of the YADH–ADP complex was used. The coenzyme dissociation constant for the NAD^+–pyrazole–YADH complex was 2.3×10^{-6} M (Karlovic et al., 1976). The dissociation constants of the binary complexes of PHLDH with NAD^+ and NADH at 25 °C and pH 7 are 3.6×10^{-4} M and 4.5×10^{-6} M, respectively (Schmid et al., 1976). The dissociation constants of oxalate and oxamate from the ternary PHLDH–NAD^+–oxalate and PHLDH–NADH–oxamate complexes are 1.4×10^{-6} M and 2.5×10^{-6} M, respectively (Schmid et al., 1976).

Exchange Studies of YADH in the Presence of an Equilibrium Mixture of Coenzymes and Substrates. The composition of a mixture containing 30.8×10^{-3} M NAD^+, 7.3×10^{-3} M NADH, 3.8×10^{-2} M acetone, and 3.9×10^{-2} M 2-propanol was found to remain unchanged for the duration of 5 h in the presence of 2×10^{-4} M YADH in D_2O, pD 8.0 at 20 °C, when measured by UV spectrophotometry between 300 and 400 nm. From the binary dissociation constants (see above), assuming four independent binding sites (Hayes & Velick, 1954), it was calculated that in this equilibrium mixture the enzyme is occupied to 60% by NADH and to 40% by NAD^+. Considering that the dissociation constants are somewhat different in the ternary complexes, these figures are only approximations. The 2-propanol and acetone used displayed no measurable absorbance in the infrared region, at these concentrations.

RESULTS

Figure 1 shows the effect of NAD^+ and NADH on the peptide hydrogen exchange of YADH at pD 8. The course of the exchange curves, which is drawn with the standard deviation, corresponds to the sum of the first-order decay functions of the individual exchangeable peptide hydrogens in the protein (Hvidt & Nielsen, 1966). It is evident that in the absence of coenzyme about 750 peptide hydrogens exchanged even before any measurement could be taken, about 190 hydrogens exchanged over a period of 300 min, and a core of 460 hydrogens remained unexchanged under these conditions.

In the presence of both coenzymes, NAD^+ and NADH, the rate of exchange is reduced as indicated by an upward displacement of the curve. Thus, at 300 min of exchange, fewer peptide hydrogens, about 28 and 75, respectively, are exchanged with NADH and NAD^+. Thus, NAD^+ has a more pronounced effect on the exchange behavior of YADH than NADH. Although in the early phase of the reaction NADH is more effective in retarding exchange, it becomes less so after 15 min where its exchange curve crosses that of the YADH–NAD^+ complex.

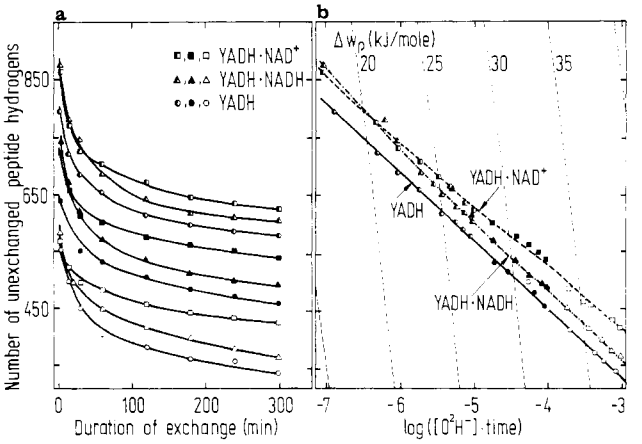

FIGURE 2: Effect of pD on the H–D exchange of YADH and YADH–coenzyme complexes. (a) [Enzyme], 2×10^{-4} M; [coenzyme], 5×10^{-2} M. Squares, YADH–NAD^+ complexes; triangles, YADH–NADH complexes; circles, YADH alone. Open symbols, pD 9.0; filled symbols, pD 8.0; half-filled symbols, pD 7.0. (b) Data shown in (a) plotted vs. the logarithm of the product of the hydroxyl ion concentration and the duration (in minutes) of the exchange reaction ("exchange relaxation spectra"). The parallel dashed lines are first-order exchange curves of hypothetical polypeptides with uniformly exchanging peptide hydrogens (eq 1), but with varying solvent accessibility. The horizontal displacements of these lines denote changes corresponding to a constant difference in transconformational free energy ΔW_{ρ_i}, where $\Delta W_{\rho_i} = -RT \ln \rho_i$. Due to the greater uncertainty in assessing the number of unexchanged peptide hydrogens at the very early stages, some of the data points obtained in the first 30 min were omitted from the plot. $[O^2H^-]$ was calculated from pD by using the ionic product of water.

Figure 2a shows that the exchange retardation by the coenzymes is maintained over the pD range 7–9 and the exchange velocities of the protein increase substantially, as manifested by the downward displacement of the family of exchange curves with increasing basicity. Clearly, the exchange retardation by the coenzymes becomes more pronounced when the pD increases. Furthermore, at pD 7 the crossover of the exchange curve of the NADH complex with that of the NAD^+ complex occurs later (40 min) than at pD 8 (15 min) and pD 9 (5 min). In Figure 2b the data of Figure 2a are replotted vs. the decadic logarithm of the product $[O^2H^-]t$ according to Willumsen (1971). As expected for most globular proteins examined with this method, the experimental points of the enzyme obtained at different times and pD values lie reasonably well on a continuous monotonically decreasing curve. The corresponding continuous monotonic curves of the enzyme–coenzyme complexes are displaced toward higher values of the log $[O^2H^-]t$ axis. The three curves are not parallel; while the curve of the YADH–NAD^+ complex diverges from the control, that of YADH–NADH is slightly convergent.

The retarding effect of the coenzyme on the H–D exchange of YADH is a function of saturation of the complex. An increase in the concentrations of free NAD^+ (Figure 3a) and NADH (Figure 3b) leads to a corresponding increase in retardation of the exchange reaction. A plot of the relative change in the number of unexchanged hydrogens, i.e., the vertical displacement of the exchange curves vs. the calculated

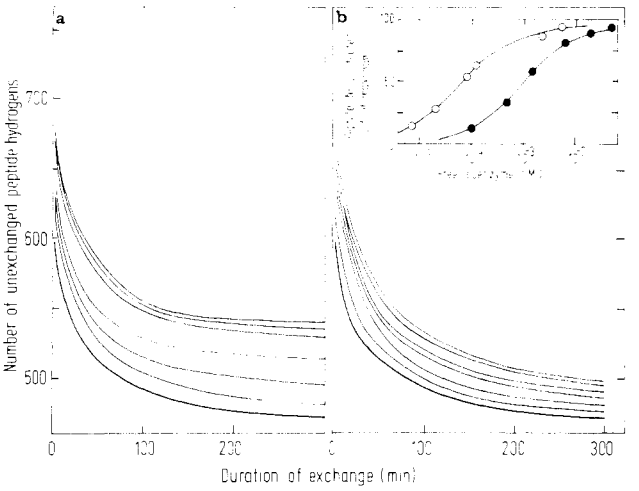

FIGURE 3: (a) Effect of NAD^+ concentration on H–D exchange of YADH. Total [NAD^+] from the bottom in ascending order: none, 2×10^{-4}, 8×10^{-4}, 2×10^{-3}, 7×10^{-3}, 2×10^{-2}, and 5×10^{-2} M. [Enzyme], 2×10^{-4} M, pD 8.0. (b) Effect of NADH concentration on H–D exchange of YADH. Total [NADH] from the bottom in ascending order: none, 2×10^{-4}, 4×10^{-4}, 7×10^{-4}, 8×10^{-4}, 3×10^{-3}, and 6×10^{-3} M. [Enzyme], 2×10^{-4} M, pD 8.0. (Inset) Increase in the number of unexchanged peptide hydrogens at 240 min in percent of maximum, plotted vs. free [NAD^+] (●) and [NADH] (○). The concentrations of free coenzyme were calculated as given under Materials and Methods.

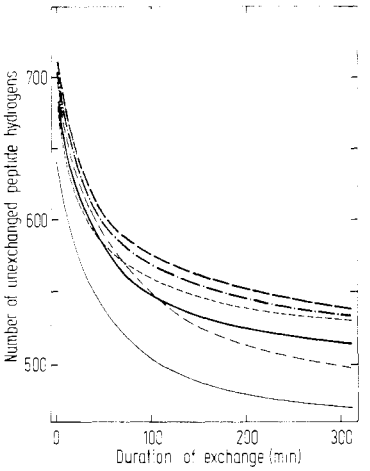

FIGURE 4: Effect of NAD coenzymes and coenzyme fragments on the H–D exchange of YADH at pD 8.0. [Ligand], 5×10^{-2} M; [enzyme], 2×10^{-4} M. Sample compositions: YADH control (—); YADH–NADH (–·–); YADH–AMP (bold —); YADH–NAD^+ (- - -); YADH–ADP (bold –·–); YADH–ADP-ribose (bold – –).

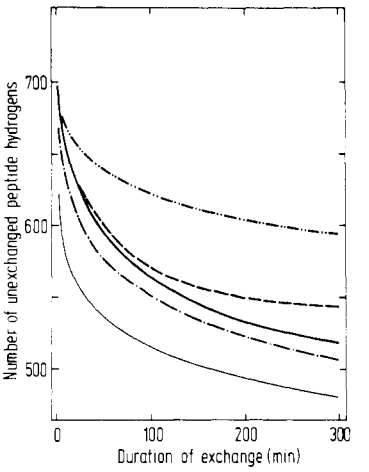

FIGURE 5: Effect of ternary complexes on the H–D exchange of YADH at pD 8.0. Sample compositions in ascending order: YADH control (—); YADH + NADH (7.3×10^{-3} M) + 2-propanol (3.9×10^{-2} M) (- - -); YADH + NAD^+ (30.8×10^{-3} M) + NADH (7.3×10^{-3} M) + 2-propanol (3.9×10^{-2} M) + acetone (3.8×10^{-2} M) (bold —); YADH + NAD^+ (30.8×10^{-3} M) + acetone (3.8×10^{-2} M) (– –); YADH + NAD^+ (5×10^{-2} M) + pyrazole (5×10^{-2} M) (–·–·–); [Enzyme], 2×10^{-4} M. The exchange curve of a sample containing YADH + NAD^+ (30.8×10^{-3} M) + NADH (7.3×10^{-3} M) was superimposable with that obtained with the equilibrium mixture. 2-Propanol and acetone alone or combined had no measurable effect on the course of H–D exchange of YADH.

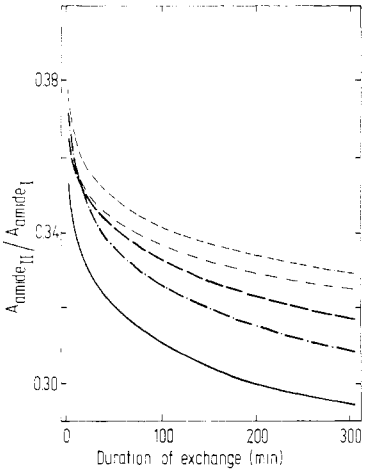

FIGURE 6: H–D exchange of PHLDH at pD 8.0. [Ligand], 5×10^{-2} M; [enzyme], 2×10^{-4} M. Sample compositions ascending order: PHLDH control (—); PHLDH + NADH (- - -); PHLDH + NAD^+ (– –); PHLDH + NADH + sodium oxamate (–··–), PHLDH + NAD^+ + potassium oxalate (– – – – –).

free coenzyme concentration, yields characteristic titration curves for the two complexes (Figure 3, inset). The inflection point observed for YADH–NADH complexes is about 5.6×10^{-5} M, a value close to that measured by spectropolarimetric titration (see Materials and Methods). A similar close fit is found for the YADH–NAD^+ complex, about 9×10^{-4} M.

The effect on the H–D exchange of YADH is even more pronounced on binding of some fragments of NAD^+. As shown in Figure 4, saturating concentrations of ADP-ribose and ADP are more effective than NAD^+, and AMP is intermediate between NAD^+ and NADH.

Binary enzyme–coenzyme complexes are often stabilized by the formation of ternary complexes with substrates, substrate analogues, and related inhibitors. Thus, the addition of pyrazole increases the affinity of YADH for NAD^+ by 2 orders of magnitude (Karlovic et al., 1976), and also influences very markedly its exchange behavior (Figure 5). Thus, in the presence of 5×10^{-2} M pyrazole, about 50 more peptide hydrogens remain unexchanged after 300 min as compared to the binary YADH–NAD^+ complex. In contrast, ternary complexes formed with the products of the enzymatic reaction (dead-end complexes), such as the YADH–NAD^+–acetone and YADH–NADH–2-propanol complexes as well as the complexes that are formed with YADH in the presence of an equilibrium mixture of NAD^+ + NADH + acetone + 2-propanol, do not show any measurable alteration of exchange behavior. The exchange curve observed in the presence of the equilibrium mixture was indistinguishable from that observed with the corresponding mixture of complexes of YADH with NAD^+ and NADH alone.

Results obtained for PHLDH (Figure 6) are comparable to those observed with binary and ternary complexes of YADH. Again binding of NAD^+ retards the exchange of the peptide hydrogens more than NADH. There is also a crossing over of the exchange curve in the early stages of the reaction. Analogous to the effect of pyrazole on the binary YADH–

NAD^+ complex, the binding of oxalate and oxamate to the binary PHLDH-NAD^+ and PHLDH-NADH complexes reduces the exchangeability of the peptide hydrogens even more. The strongest retardation is observed in the PHLDH-NAD^+-oxalate complex at 300 min of exchange.

Discussion

The results presented here confirm and extend the previous observations (Hvidt & Kägi, 1963) that binding of NAD coenzymes to YADH induces discrete changes in the rates with which the peptide hydrogens of the enzyme exchange with the solvent. This change manifests itself as a retardation of exchange which is evident from the upward displacement of the exchange curves following coenzyme binding (Figure 1). More significantly, in accordance with our earlier data, the retardation of exchange rate is larger for NAD^+ bound to the enzyme than for NADH, suggesting a greater protective effect of the former on the enzyme than of the latter.

In the set of experiments carried out in this study, at least 40% of all hydrogens exchange very rapidly with deuterium and at least 25% remain unexchanged within the period of observation (Figure 1). Thus, the measurements are restricted to the exchange behavior of at most 35% of all peptide hydrogens. A difficulty in all these studies is that the effects of ligand binding on the exchange velocity are reflected only in a relatively small displacement of the exchange curve of the complex from that of the unliganded protein. In YADH, the vertical separation of the exchange curve of the NAD^+ complex from that of the control is about 70 peptide hydrogens or 5% of the total, and the difference is even smaller for the NADH complex. The measurement of such small differences puts great demands on experimental precision. The infrared spectrophotometric method, which has the advantage of speed and simplicity and approaches the precision attained by UV spectrophotometry, is especially suitable for such comparative studies. It allows the measurement of the number of unexchanged peptide protons with a reproducibility of ±6 hydrogens, if the exchange reaction is carried out on samples of the same batch of enzyme and lyophilized simultaneously from the same solution, as in the present case.

Among the predominant mechanisms invoked to analyze solvent exchange data is the one according to Linderstrøm-Lang (Hvidt & Nielsen, 1966), in which the nonexchanging folded and the exchanging locally unfolded conformations are in equilibrium with each other. In the absence of pH-induced conformational changes, two limiting cases that relate the rate of exchange with transconformational processes can be experimentally differentiated. For the case in which solvent exchange is the rate-limiting step (the EX-2 mechanism or high-motility model), the number of unexchanged peptide hydrogens x at time t is given by

$$x = \sum_{i=1}^{n} e^{-\rho_i k_0 [O^2H^-] t} \qquad (1)$$

where ρ_i is a conformation-dependent equilibrium constant determining the solvent accessibility of peptide hydrogen i and k_0 is the average pH-independent bimolecular exchange rate constant (Hvidt & Nielsen, 1966; Willumsen, 1971). Therefore, when k_0 is constant, i.e., at constant temperature, alterations in exchange rate at a given basicity $[O^2H^-]$ can arise only from shifts in the conformational equilibria ρ_i.

For a number of globular proteins, cytochrome c (Kägi & Ulmer, 1968), aspartate aminotransferase (Pfister et al., 1985), and others (Willumsen, 1971; Ottesen, 1971; Hvidt & Wallevik, 1972; Mizuta et al., 1980; Roder et al., 1985), the validity of the EX-2 mechanism has been demonstrated and the consequent correlation of changes in exchange rates with changes in conformational equilibria established. For YADH, therefore, as a first approximation, the exchange retardation (Figure 1) due to coenzyme binding is likely to reflect not merely a static protective effect on the enzyme as suggested earlier (Hvidt & Kägi, 1963) but a pronounced change in conformational equilibrium. The present data document that this effect is a direct function of binding site occupation by the two coenzymes (Figure 3, inset).

The kinetic curves of Figure 1 clearly highlight the differences in the exchange retardation caused by either coenzyme. NADH initially exerts a slightly greater influence on the faster exchanging hydrogens than does NAD^+, but as exchange proceeds, this order is reversed. This is indicated by the presence of a crossover point between the two curves which was also shown previously (Hvidt & Kägi, 1963) and which suggests that distinctly different conformational forms of the enzyme are stabilized by the binding of NADH and NAD^+. The unequal effects of coenzyme binding on the exchangeability of the more exposed peptide hydrogens monitored in the initial phase of the exchange experiments and of the more buried peptide hydrogens monitored in the later phases are presumably related to their unequal topographic location in the protein.

That the EX-2 mechanism is indeed operational in the case of YADH and its coenzyme complexes is demonstrated by the pH studies shown in Figure 2a. In accordance with the dependence of the negative exponent in eq 1 on deuterioxide concentration, the family of exchange curves are displaced downward with increasing basicity and the crossover points of the curves due to the coenzymes are shifted to earlier times. This downward shift indicates that, at higher basicity, peptide protons that were not exchanged at lower pD, and that presumably are less exposed to solvent, are now monitored. The persistence of exchange retardation between the free and complexed enzyme forms with increasing pD is thus a demonstration that coenzyme binding is felt globally by the protein.

Graphical documentation for the high-motility model for YADH and its coenzyme complexes is obtained from an analysis of the data of Figure 2a according to Willumsen (1971). When these data are replotted (Figure 2b) on a decadic logarithmic scale against the product $[O^2H^-]t$ in accordance with eq 1, three continuous monotonically decreasing functions are obtained, illustrating that the exchange behavior of both coenzyme-bound and of free YADH conforms to the EX-2 scheme. The data for each form of the enzyme span 4 log units (Figure 2b). Since first-order decay functions decrease to about 5% of the initial value within 2 log units, at least two nonoverlapping classes of peptide hydrogens, exchanging with rate constants differing by 10^2, are being monitored in these experiments (Ottesen, 1971).

The logarithmic plots ranking the peptide hydrogens according to their decreasing exchange rates have been designated as exchange relaxation spectra (Zavodszky et al., 1975). The mutual displacement of these relaxation spectra is a direct demonstration of conformation differences between the three forms of YADH examined. Paralleling the trends in the decay curves (Figures 1 and 2a), the slopes of the relaxation spectra of the coenzyme-bound forms differ somewhat from that of the free enzyme. The curve of the YADH-NAD^+ complex diverges from that of the control while that arising from the YADH-NADH complex converges, resulting in the early crossover point commented on above. With acceptance of the validity of the EX-2 scheme for the systems under study (see above), the extent of the displacement in the relaxation

spectrum (Figure 2b) offers some measure of the free energy change associated with the conformational transition (Hvidt & Nielsen, 1966; Kägi & Ulmer, 1968; Englander et al., 1982). Thus, from the displacement of the exchange curve of the YADH–NAD^+ complex from that of YADH, it follows that the monitored peptide hydrogens are retarded by a factor ranging from 2.4 to 5.5 early and late in the course of exchange, respectively. According to eq 1, this can be related to a corresponding change in transconformational equilibrium constants ρ_i and hence to the net difference in transconformational free energy ($\Delta W\rho_i$) ranging from 2.3 to 4.3 kJ/mol, respectively, between equally ranking peptide hydrogens. For the complex with NADH the corresponding retardation factors range from 2.8 to 1.5, which leads to differences in $\Delta W\rho_i$ with respect to the corresponding peptide hydrogens in the free enzyme of 2.6 and 1.5 kJ/mol, respectively. From the average values of the changes in $\Delta W\rho_i$ (Figure 2b), it is obvious that the effect of NAD^+ ($\Delta W\rho_{av}$ = 3.3 kJ/mol) is significantly larger than that of NADH ($\Delta W\rho_{av}$ = 2 kJ/mol).

As we elaborated earlier (Kägi & Ulmer, 1968), the observed values for $\Delta W\rho_{av}$ give only minimum estimates of the transconformational work expended by the coenzyme[2] in changing the ρ_is in enzyme–coenzyme complex formation. $\Delta W\rho_{av}$ is an accurate measure of this work only in the hypothetical case in which binding of the coenzyme affects uniformly and coordinately all exchangeable peptide hydrogens. To the extent that this condition is not met (see above) and considering the possibility that coenzyme binding might in fact accelerate rather than retard the exchange of some peptide hydrogens, the actual free energy difference separating the transconformational equilibria displaced by ligand binding would be correspondingly larger. As implicit in the original formulation of the high-motility model of Linderstrøm-Lang, these "microscopic" transconformational free energy differences relate to changes in global conformational equilibria of the protein. Hence, the observed differences in the exchange rates provide an unambiguous albeit semiquantitative energetic measure of the extent of the global conformation changes induced by coenzyme binding in YADH.

The overall strong retarding effect of NAD^+ as compared to that of NADH is repeated in the exchange behavior of their complexes with PHLDH (Figure 6). Similar effects have been observed on complex formation of these coenzymes with CHLDH[1] (DiSabato & Ottesen, 1965), GAPDH[1] (Zavodsky et al., 1966), and GDH[1] from bovine liver (Stryker & Parker,1970), suggesting that it is a general characteristic of complexes of dehydrogenases with pyridine nucleotides. in each case, the unequal exchange retardation thus reinforces that the two coenzymes impose specific constraints that lead to quite different conformations. This is substantiated in LADH and DFLDH also by X-ray crystallographic data [Eklund & Brändén, 1979; Adams et al. (1973) and references cited therein].

The exertion of conformational constraints by the coenzymes upon the enzymes is compatible with the concept of the selective stabilization of a particular set of conformers out of an ensemble of essentially degenerate substates constituting the free enzyme (Steitz et al., 1983; Frauenfelder, 1983). Evidently then, NAD^+ and NADH select substantially different sets of conformers in the dehydrogenases examined thus far. In principle, such a partitioning of the ensemble of substates by ligand binding can lead to either a reduction or an enhancement of the peptide hydrogen exchange rates, depending on the average compactness of the sets of conformers selected. In most known cases ligand binding leads to retardation, but as exemplified by O_2 and CO binding to hemoglobin, exchange can also be facilitated (Hedlund et al., 1978; Ghose & Englander, 1974).

The exchange patterns displayed by the coenzyme fragments (Figure 4) are also explicable in terms of the same arguments. The surprisingly strong retarding effects of ADP-ribose and ADP thus suggest the selection of a population of more slowly exchangeable conformers. The fact that the net charges on ADP-ribose and NADH are the same but the exchange retardations are radically different implies that charge alone is not a major determinant in these ligand-mediated effects on YADH. The further increase in retardation of the H–D exchange reaction on the combined binding of NAD^+ and pyrazole (Figure 5) to YADH and of NAD^+ and oxalate and NADH and oxamate (Figure 6) to PHLDH shows that ternary complex formation imposes conformational tightening on the enzyme above that seen with the binary enzyme–coenzyme complexes. These dead-end complexes approximate the transition state (Subramanian, 1979), and hence, the enhanced exchange retardations may be compared to those observed for the productive intermediates of catalysis in aspartate aminotransferase (Pfister et al., 1985).

In contrast to the above transition-state analogue complexes of YADH–NAD^+ with pyrazole, both the dead-end complexes of YADH with NAD^+ and acetone and those with NADH and 2-propanol, as well as the mixture of dead-end and productive complexes presumably formed in the equilibrium mixture of pairs of coenzyme and substrate, display no exchange retardation above that exhibited by the corresponding mixture of the respective binary YADH–NAD^+ and YADH–NADH complexes. This may be expected since, to our knowledge, true intermediates of catalysis in pyridine nucleotide dependent dehydrogenases have not been documented.

The most conspicuous observation in this study with YADH and PHLDH is that NAD^+ with its higher dissociation constant ($K_D^{YADH} = 6.7 \times 10^{-4}$ M, $K_D^{PHLDH} = 3.6 \times 10^{-4}$ M) is distinctly more effective in retarding exchange than NADH ($K_D^{YADH} = 2.5 \times 10^{-5}$ M, $K_D^{PHLDH} = 4.5 \times 10^{-6}$ M). In view of the correlation made above between retardation and shifts in conformational equilibria, it is logical to attribute the unequal affinities primarily to the unequal conformational work expended on the protein by the coenzymes. Janin and Chothia (1978) have inferred from the loss of accessible surface area that hydrophobicity makes the dominating contribution to the binding of the coenzymes to dehydrogenases. The hydrophobic free energy gain calculated from the estimated total surface area reduction upon complex formation of DFLDH and an NAD coenzyme (−1222 Å^2) is on the order of −30 kcal/mol. Of this total free energy gain 20–22 kcal/mol is expended for work compensating for the loss of translational and rotational degrees of freedom, leaving about −8 to −10 kcal/mol for the net free energy gain of the protein–ligand interaction. Our data imply that the latter term can be further subdivided into an actual binding term, $-\Delta G_b$, and that expended for the accompanying conformational change, proportional to $\Delta W\rho_{av}$. Thus, on the reasonable assumption that for a particular dehydrogenase both the large total hydrophobic free energy change and the contributions to translational and rotational entropic terms are closely similar for NAD^+ and NADH, it follows that there will be an opposing variation in binding strength and the extent of conformational change. This trend is apparent in the data summarized in Figure 7 for

[2] The sign of $\Delta W\rho_i$ is taken as positive for all changes in ρ_i attendant upon coenzyme binding.

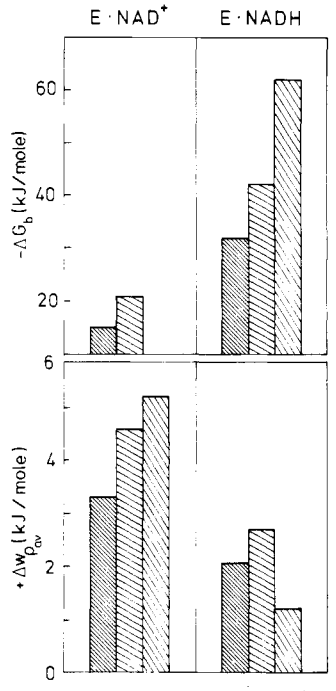

FIGURE 7: Plot showing the opposing variation between free energy of binding $-\Delta G_b$ and conformational work $+\Delta W\rho_{av}$ due to NAD^+ and NADH binding to YADH (left bar), PHLDH (center bar), and CHLDH (right bar). $\Delta W\rho_{av}$ was calculated from the average exchange retardation obtained from the data shown in Figures 2b and 6 and published data on CHLDH (DiSabato & Ottesen, 1965), respectively. $-\Delta G_b$ for complexes with YADH and PHLDH were calculated from the binding constants at 25 °C (see Materials and Methods). The value of $-\Delta G_b$ for the CHLDH-NADH complex is from McKay and Kaplan (1960).

both YADH and PHLDH examined in this work and CHLDH reported in an independent study (DiSabato & Ottesen, 1965). Thus, in all three instances complexation with NAD^+ is associated with an appreciably larger expenditure of conformational work.

Correlations made earlier for several dehydrogenases between thermodynamic binding terms and molecular features like charge and size of NAD coenzymes and their fragments have not been conclusive [Beaudette and Langerman (1980) and references cited therein]. Particularly, the reason for the frequently encountered lower affinity of NAD^+ for dehydrogenases catalyzing the oxidation of alcohols, i.e., YADH, LADH, LDH,[1] MDH,[1] GDH, and isocitrate dehydrogenase, is not clear. The present data supply an operational principle to explain the differential affinities of the two coenzymes for these enzymes. Clearly, as discussed above, the unequal expenditure of conformational work linked to binding offers a means of modulating the binding constants. The fine tuning of the latter was long ago claimed to be an essential requirement for the optimization of the redox function of these enzymes (Theorell & Bonnichsen, 1951) and is undoubtedly of paramount importance for their integration in the metabolic network. In a similar study we have previously implicated a mechanistic involvement of conformational equilibria in the critical setting of the redox potential of cytochrome *c* (Kägi & Ulmer, 1968). The overriding importance of an accurate scaling of the binding constants of binary and ternary complexes to catalytic efficiency was recently also documented in genetically engineered tyrosyl-transfer RNA synthetases (Fersht et al., 1986). It is thus conceivable that through the introduction of selective and discrete conformational constraints dehydrogenase function has been perfected in evolution.

Registry No. YADH, 9031-72-5; PHLDH, 9001-60-9; NAD, 53-84-9; NADH, 58-68-4; ADP, 58-64-0; AMP, 61-19-8; ADP-ribose, 20762-30-5; pyrazole, 288-13-1; oxalate, 144-62-7; oxamate, 471-47-6.

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Classical Raman Spectroscopic Studies of NADH and NAD^+ Bound to Liver Alcohol Dehydrogenase by Difference Techniques†

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ABSTRACT: We report the Raman spectra of reduced and oxidized nicotinamide adenine dinucleotide (NADH and NAD^+, respectively) and adenosine 5′-diphosphate ribose (ADPR) when bound to the coenzyme site of liver alcohol dehydrogenase (LADH). The bound NADH spectrum is calculated by taking the classical Raman difference spectrum of the binary complex, LADH/NADH, with that of LADH. We have investigated how the bound NADH spectrum is affected when the ternary complexes with inhibitors are formed with dimethyl sulfoxide (Me_2SO) or isobutyramide (IBA), i.e., LADH/NADH/Me_2SO or LADH/NADH/IBA. Similarly, the difference spectra of LADH/NAD^+/pyrazole or LADH/ADPR with LADH are calculated. The magnitude of these difference spectra is on the order of a few percent of the protein Raman spectrum. We report and discuss the experimental configuration and control procedures we use in reliably calculating such small difference signals. These sensitive difference techniques could be applied to a large number of problems where the classical Raman spectrum of a "small" molecule, like adenine, bound to the active site of a protein is of interest. The spectrum of bound ADPR allows an assignment of the bands of the bound NADH and NAD^+ spectra to normal coordinates located primarily on either the nicotinamide or the adenine moiety. By comparing the spectra of the bound coenzymes with model compound data and through the use of deuteriated compounds, we confirm and characterize how the adenine moiety is involved in coenzyme binding and discuss the validity of the suggestion that the adenine ring is protonated upon binding. The nicotinamide moiety of NADH shows significant molecular changes upon binding. We find that the aromatic nature of the NAD^+ nicotinamide ring is disrupted in the ternary complex LADH/NAD^+/pyrazole. We discuss various models which are consistent with the data and with the enzymatic mechanism of LADH. We finally note that the rather dramatic changes in the coenzyme molecular structure, that occur when NADH or NAD^+ binds, are not necessarily repeated at other dehydrogenase binding sites.

Nicotinamide adenine dinucleotides (NAD^+ and NADH)[1] are coenzymes for hundreds of oxidation-reduction reactions (Daziel, 1975). Their roles in enzymatic reactions have been under investigation for the last few decades by various techniques (Kaplan, 1960; Colowick et al., 1966; Hollis, 1967; Fisher et al., 1969; Schlessinger et al., 1975; Subramanian &

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[1] Abbreviations: NAD^+, oxidized β-nicotinamide adenine dinucleotide; NADH, reduced β-nicotinamide adenine dinucleotide; LADH, liver alcohol dehydrogenase; ADPR, adenosine 5′-diphosphate ribose; AMP, adenosine 5′-monophosphate; NMN, β-nicotinamide mononucleotide; IBA, isobutyramide; Me_2SO, dimethyl sulfoxide; OMA, optical multichannel analyzer; fwhm, full width at half-maximum.

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